Neuroscience 118 (2003) 323–334

THE CYCLIN-DEPENDENT KINASE 5 ACTIVATOR, p39, IS EXPRESSED IN STRIPES IN THE MOUSE CEREBELLUM

Y.-G. JEONG,[1] J. L. ROSALES, H. MARZBAN, R. V. SILLITOE, D.-G. PARK, R. HAWKES AND K.-Y. LEE*

[a]*Department of Cell Biology and Anatomy, The University of Calgary, Calgary, Alberta, Canada T2N 4N1*

Abstract—Cyclin-dependent kinase 5 (Cdk5) activity is required for CNS development. The Cdk5 activator, p35, is well characterized but its isoform, p39, has been less studied. Previously, p39 mRNA expression in rat brain was shown to peak at 3 weeks postnatal, and the level remains high in the adult cerebellum [Neurosci Res 28 (1997) 355]. However, p39 protein expression and specific localization in the cerebellum, where p39 mRNA level significantly exceeds that of p35, have not been examined. Here, we explored the specific cerebellar localization of the p39 protein in the developing and adult mice. Adult cerebellar Purkinje cell somata and dendritic arbors were strongly positive for p39 but only rare and barely detectable p39 was observed in Purkinje cell axons. Cdk5 also localized in Purkinje cell somata and dendrites of the adult cerebellum, but p35 localized only in Purkinje cell somata, further suggesting a functional difference between p35 and p39. During development, cerebellar p39 was first noted at P10. Primary cultures of a developing cerebellum also showed strong p39 immunoreactivity in Purkinje cell somata and dendrites, but weak p39 immunoreactivity in Purkinje cell axons. Starting from P10, p39 was observed in a subset of Purkinje cells that form parasagittal bands throughout the vermis and hemispheres. These bands were bilaterally symmetrical and continuous from one lobule to another. Conversely, Cdk5 and p35 showed a uniform staining pattern. The pattern of p39 closely resembled that of zebrin II/aldolase C, suggesting that p39 may play a role in the adult cerebellum rather than in pattern development. This premise is consistent with the normal pattern of zebrin II/aldolase C zones and stripes in mutant p39−/− mice. The alternating p39 parasagittal band pattern may reflect a role for p39 or Cdk5/p39 in the functional compartmentation of the cerebellum.

Key words: Purkinje cells, parasagittal bands, p35 isoform.

Cyclin-dependent kinase 5 (Cdk5) was originally identified based on its primary sequence homology to the key regulators of eukaryotic cell-cycle progression, Cdc2 and Cdk2 (Morgan, 1997). However, unlike other Cdks, Cdk5 activity is primarily associated with post-mitotic neurons and differentiated cell functions such as neuronal migration (Homayouni and Curran, 2000), axon patterning (Connell-Crowley et al., 2000), and secretion (Fletcher et al., 1999). Cdk5 activity has also been implicated in neurodegenerative conditions, particularly in Alzheimer's disease (Lee et al., 1999; Patrick et al., 1999). Indeed, Cdk5 has been shown to phosphorylate tau on sites that are abnormally phosphorylated in the paired helical filaments characteristic of Alzheimer's-disease brains (Paudel et al., 1993). In addition, tau phosphorylation by Cdk5 promotes tau dimerization *in vitro* (Paudel, 1997). For its activity, Cdk5 requires the neuronal Cdk5 activator, p35 (Lew et al., 1994, Tsai et al., 1994) or its isoform, p39 (Tang et al., 1995). The p39 isoform shares a 57% amino acid homology with p35 and can also activate Cdk5 (Tang et al., 1995). *In situ* hybridization studies revealed that p35 and p39 mRNAs are expressed predominantly in the CNS (Lew et al., 1994; Tsai et al., 1994; Uchida et al., 1994; Tang et al., 1995; Cai et al., 1997; Zheng et al., 1998; Ohshima et al., 2001). Both p35 and p39 transcripts are first detected at embryonic day 12 (E12) and mRNA expression increases substantially by E15–E17 (Zheng et al., 1998). However, while p35 mRNA expression is highest in the newborn rat brain (Uchida et al., 1994; Tomizawa et al., 1996), p39 mRNA expression peaks postnatally at 3 weeks, and p39 mRNA levels remain particularly high in the cerebellum of the adult (Cai et al., 1997). These findings suggest distinct functions for p35 and p39 during brain development. At the protein level, p35 and p39 have been localized to both the cerebral and the cerebellar cortices by immunohistochemical staining (Matsushita et al., 1996; Honjyo et al., 1999) and Western blot analysis (Humbert et al., 2000; Wu et al., 2000). While subcellular localization studies have shown that p35 is consistently found in neuronal somata during cerebellar development (Matsushita et al., 1996; Tomizawa et al., 1996), there are no detailed reports on the specific localization of p39 in the cerebellum. In this study, we explored the specific cerebellar localization of the p39 protein in the developing and adult mice.

Despite the apparent uniformity of the adult cerebellar cortex, it can be subdivided structurally, molecularly, and functionally into transverse zones and parasagittal stripes (Hawkes and Eisenman, 1997; Herrup and Kuemerle, 1997; Oberdick et al., 1998; Ozol et al., 1999; Armstrong and Hawkes, 2000), and its complex heterogeneous pattern can be revealed by using biochemical markers. For example, zebrin II/aldolase C, the most studied molecular marker, is expressed by a subset of Purkinje cells forming

[1] Present address: Department of Anatomy, College of Medicine, Konyang University, 26 Naedong, Nonsan City, Chungnam, 320-711, South Korea.

*Corresponding author. Tel: +1-403-220-8723; fax: +1-403-270-0834.

E-mail address: kylee@ucalgary.ca (K.-Y. Lee).

Abbreviations: Cdk5, cyclin-dependent kinase 5; E, embryonic day; EDTA, ethylenediaminetetraacetic acid; HEPES, HCO(3-)-free *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; PB, phosphate buffer; PBS, phosphate-buffered saline; P, postnatal day; RT, room temperature; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TPBS, Tris–phosphate-buffered saline.

0306-4522/03$30.00+0.00
doi:10.1016/S0306-4522(03)00002-2

an array of stripes and zones that are highly reproducible between individuals (Brochu et al., 1990; Ahn et al., 1994; Armstrong and Hawkes, 2000; Oberdick et al., 1998). Bands of zebrin II/aldolase C immunoreactive Purkinje cells in the cerebellum are numbered from P1+ at the midline to P7+ laterally in the hemispheres, and zebrin II non-immunoreactive bands are numbered from P1− to P7− accordingly (Eisenman and Hawkes, 1993). The basic topography of the mouse cerebellar vermis consists of a series of transverse zones: the anterior zone (lobules I–V), central zone (lobules VI–VII), posterior zone (lobules VIII–IX) and the nodular zone (lobule X) (Hawkes and Eisenman, 1997; Ozol et al., 1999). Zebrin II/aldolase C is expressed uniformly in the central and nodular zones, and in parasagittal stripes of Purkinje cells in the anterior and posterior zones. A similar alternation of homogeneous and striped expression domains is seen in the hemispheres. In this report, we show that p39 is strongly localized to the somata and dendritic arbors of Purkinje cells in the developing and adult mouse cerebellum, and that the p39-immunoreactive Purkinje cells form a pattern of parasagittal bands corresponding to those revealed by zebrin II/aldolase C.

EXPERIMENTAL PROCEDURES

Animals

All experimental procedures on mice were carried out in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals (NIH publication no. 80-23), revised 1996. All efforts were made to minimize the number of animals used and their suffering. Postnatal day (P) 5, P10, P15, and P20 and adult CD1 mice were obtained from Charles River Laboratories (St. Constant, PQ, Canada). The p39−/− mice were obtained from Dr. Li-Huei Tsai at Harvard University.

Antibodies

Polyclonal p39-specific antibody was raised against a peptide corresponding to amino acid residues 329–352 at the C-terminus of the protein (Tang et al., 1995). This region is specific to p39 and has a high degree of sequence identity (70%) to its mouse counterpart (Nilden et al., 1998). The peptide was conjugated to keyhole limpet hemocyanin by using disuccinimidyl suberate. The conjugate was emulsified with Freund's adjuvant and injected intradermally into the rabbit every 2 weeks for 4 months. The antibody was purified on protein A-sepharose beads and the specificity of the antibody was evaluated by Western blot analysis. Polyclonal p35-specific and Cdk5-specific antibodies were obtained from Santa Cruz Biotech (Santa Cruz, CA, USA). Monoclonal anti-zebrin II was produced from a mouse immunized with a crude cerebellar homogenate of the weakly electric fish *Apteronotus* (Brochu et al., 1990).

Perfusion and sectioning

Mice were deeply anesthetized with sodium pentobarbital (60 mg/kg body weight, i.p.) and transcardially perfused with 0.9% NaCl in 0.1-M phosphate buffer (PB, pH 7.4) followed by approximately 100 ml of 4% paraformaldehyde in 0.1-M PB. Brains were then isolated and placed immediately in the same fixative for 24 h at 4 °C. The post-fixed brains were cryoprotected in 10%, 20% and 30% sucrose in 0.1-M PB. Cerebella were then cryostat sectioned in the frontal or sagittal planes (20- and 40-μm thickness).

Immunohistochemistry

Cerebellar sections were rinsed in 0.1-M phosphate-buffered saline (PBS, pH 7.4) containing 0.5% hydrogen peroxide for 30 min followed by 0.1-M Tris–PBS (TPBS, pH 7.7, containing 8.5-mM Na_2HPO_4, 3-mM KH_2PO_4, 125-mM NaCl, 30-mM Tris–HCl and 0.03-mM NaN_3) for 15 min. The tissue sections were then incubated overnight at room temperature (RT) in p39-specific (1:100), p35-specific (1:200), or Cdk5-specific (1:500) antibody in blocking solution (0.1-M PB, pH 7.4, containing 10% normal goat serum, 1% bovine serum albumin, 0.3% Triton X-100). The cerebellar sections were then washed five times for 6 min each in 0.1-M TPBS and incubated for 3 h at RT in peroxidase-conjugated secondary antibody (goat anti-rabbit IgG, Pierce Inc., Rockford, IL, USA) diluted 1:100 in TPBS. After three rinses in TPBS, immunoreactive sites were visualized by using a 10-min incubation in 0.2% diaminobenzidine–4HCl in TPBS. The tissue sections were further rinsed, attached onto gelatin coated slides, air-dried overnight, dehydrated by incubation in increasing concentrations of alcohol (70%, 95%, and two consecutive incubations in 100%), cleared in Histoclear (National Diagnostics, Mississauga, ON, Canada), and mounted using DPX mounting medium (BDH Chemicals, Toronto, ON, Canada).

Whole-mount immunohistochemistry

Animals were perfused as described above and whole-mount immunostained using a slightly modified protocol originally designed for screening mutations in the mouse cerebellum (Sillitoe and Hawkes, 2002). After incubating the cerebellum in fixative for 24–48 h, it was post-fixed overnight at 4 °C in Dent's fixative (Dent et al., 1989). Next the cerebellum was incubated in Dent's bleach (Dent et al., 1989) for approximately 8 h then dehydrated in 2×30 min each 100% MeOH. The tissue was passed through four to five cycles of chilling to −80 °C and thawing to RT in 100% MeOH followed by overnight incubation in MeOH at −80 °C. For zebrin II staining, the cerebellum was rehydated for 90 min each in 50% MeOH, 15% MeOH, and PBS then enzymatically digested in 10 μg/ml proteinase K (>600 units/ml; Boehringer Mannheim, Laval, Quebec, Canada) in PBS for 5 min at RT. After rinsing 3×10 min in PBS, the tissue was incubated in blocking buffer (Davis, 1993) for 6–8 h at RT. The tissue was then incubated for 48-96 h in zebrin II antibody, rinsed 3×2 h at 4 °C, and incubated for 48–72 h at 4 °C in secondary antibody (Jackson ImmunoResearch Laboratory, West Grove, PA, USA). Finally, the tissue was rinsed 4×3 h each at 4 °C followed by a final overnight rinse, incubated in PBT (Davis, 1993) for 2 h at RT, and antibody binding sites were revealed with diaminobenzidine (DAB).

Photomicrographs

Photomicrographs were captured using a Photometrics Quantix digital camera. Images were assembled using Adobe Photoshop 5.0.

Preparation of mouse whole brain/cerebellar homogenate

Adult CD1 mice whole brains or cerebella were homogenized in buffer A (25-mM HEPES, pH 7.2, containing 0.15-M NaCl, 1-mM EDTA, 1-mM dithiothreitol, 1-mM phenylmethylsulfonyl fluoride, 1 μg/ml each of leupeptin and aprotinin, 2 μg/ml of antipain, 0.3 mg/ml benzamidine). The homogenates were centrifuged at 21,000×*g* for 20 min at 4 °C and the supernatant was designated as either brain or cerebellar extract.

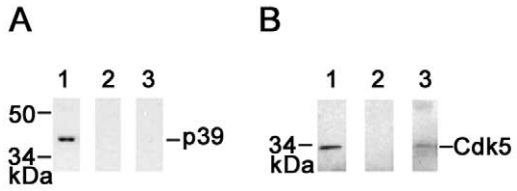

Fig. 1. Specificity of the p39 antibody. (A) Samples (50 μg each) of wild-type (lanes one and 2) or mutant p39−/− (lane 3) mouse-brain extracts were resolved in a 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotted with the p39 antibody (lanes 1 and 3) that we developed or p39 antibody that was preadsorbed with the peptide antigen used to generate the antibody (lane 2). (B) p39 was immunoprecipitated from cerebellar extracts (300 μg) using the p39 polyclonal antibody. Coimmunoprecipitation of Cdk5 was determined using a Cdk5 monoclonal antibody (DC-17, Santa Cruz Biotech) for Western blotting. Lane 1, whole-cerebellar extract; lane 2, p39 antibody was preadsorbed with the peptide antigen prior to immunoprecipitation; lane 3, p39 immunoprecipitate.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

SDS-PAGE (12.5% vertical slab gel) was performed according to the method of Laemmli (1970). Samples analyzed by SDS-PAGE were transferred to a nitrocellulose membrane (Millipore, Bedford, MA, USA) and probed with the indicated antibody (to p39, p35, or Cdk5) followed by incubation with a horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford, IL, USA), and developed using ECL reagents as specified by the manufacturer (Amersham Life Science, Inc., Buckinghamshire, England).

RESULTS

Specificity of p39 antibody

The specificity of the p39 antibody was determined by Western blot analysis of mouse brain extracts. As shown in Fig. 1A, a p39 antibody immunoreactive band, corresponding to 39 kDa, was detected in wild-type mouse brain (lane 1). The band was not detected when the p39 antibody was preadsorbed with the p39-specific peptide antigen used to develop the antibody (lane 2). Specificity of the antibody was further confirmed by the absence of an immunoreactive band in mutant p39−/− mouse brain (lane 3). By immunoprecipitation using the p39 antibody, we also show evidence for coimmunoprecipitation of Cdk5 (Fig. 1B, lane 3).

Cerebellar expression of p39 in the adult mouse

Since the p39 mRNA has been shown to be expressed strongly in the 3-week-old rat brain, and high expression is maintained in the adult cerebellum, we sought to analyze the specific localization of the p39 protein in the adult mouse cerebellum. Immunochemical staining of both cerebellar tissue sections and cerebellar cell cultures grown for 3 weeks *in vitro* yielded consistent localization of p39. In cerebellar sections, Purkinje cells in the vermis were immunoreactive for p39 with reaction product deposited throughout the somata and dendritic arbors (Fig. 2A). However, axonal staining was barely detectable. No somatic profiles of basket and stellate cells were stained in the molecular layer (Fig. 2A, D, E). In the granular layer, weak

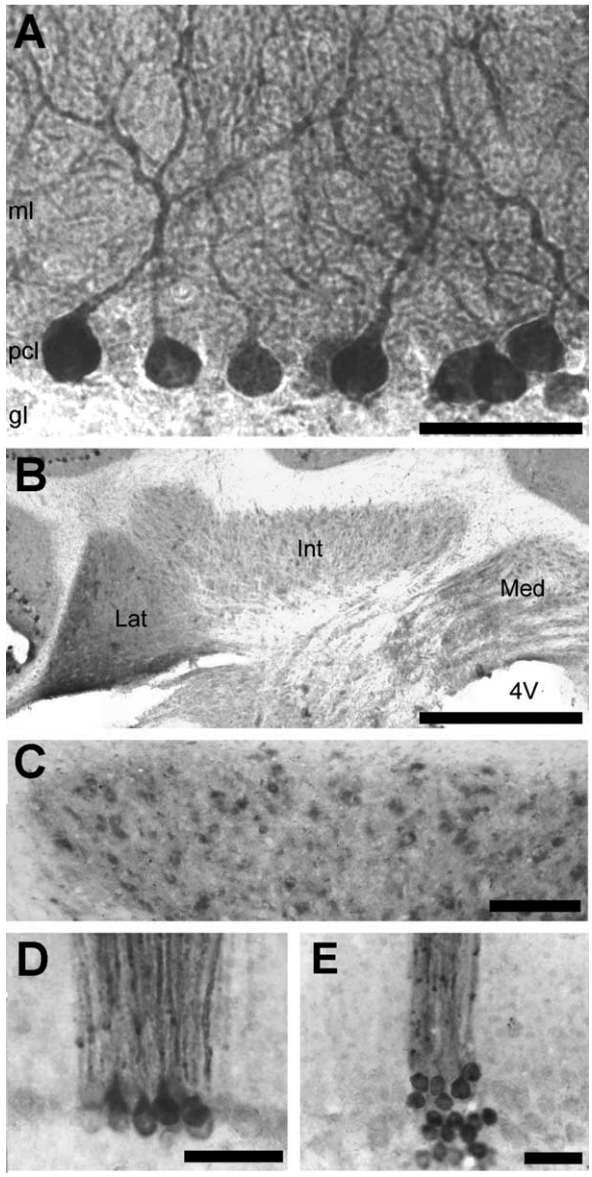

Fig. 2. Specific localization of p39 in the adult mouse cerebellar vermis. Transverse sections of the cerebellar vermis were immunostained with the p39-specific antibody as described in Experimental Procedures. (A) Immunoreactivity for p39 was found predominantly in the Purkinje cells where both the dendrites in the molecular layer (ml) and the somata in the Purkinje cell layer (pcl) were stained. Light immunoreactivity was also associated with the granule cells in the granular layer (gl). (B) Immunoreactivity was also seen in the cerebellar nuclei (Lat=lateral; Int=interposed; Med=medial; 4V=fourth ventricle). (C) The anterior interposed nucleus of the cerebellar nuclei at higher magnification is shown. (D and E) Not all Purkinje cells express p39. Bands of immunostained Purkinje cells alternating with unreactive bands are illustrated in the posterior vermis (lobule IX; D) and anterior lobe vermis (lobule V; E). Each scale bar=100 μm.

p39 immunoreactivity was observed in the granule cells but neither the Golgi epithelial cells nor their Bergmann glial fibers were stained. In addition, no p39 immunoreactivity was associated with either oligodendrocytes in the white matter or astrocytes (data not shown). In the cerebellar nuclei (Fig. 2B), large neuronal somata were labeled

with p39 (Fig. 2C). Interestingly, p39 immunoreactivity was not present in all Purkinje cells (Fig. 2D, E). Instead, transverse sections of the cerebellum revealed that p39-immunoreactive Purkinje cells form bands that alternate with bands of p39 unreactive cells. The distribution of p39 in primary cultures (Fig. 3) from newborn mouse cerebellum maintained 3 weeks *in vitro* supports our observations in cerebellar tissue sections. Double-label immunofluorescence for calbindin (Fig. 3A, D) and p39 (Fig. 3B, E) showed that many Purkinje cells, the only cerebellar neurons that express calbindin (Celio, 1990; Ozol et al., 1999), express p39 both in their somata and dendritic arbors. As found *in vivo*, the putative Purkinje cell axon was weakly immunoreactive to p39 (Fig. 3B, C). In addition, as seen in tissue sections, p39 immunoreactivity was also present in numerous small neurons (identified by double labeling with anti-MAP2, data not shown), which appear to be granule cells (Fig. 3B). Finally, and again consistent with the results obtained from immunohistochemical analysis, a subset of Purkinje cells (Fig. 3D–F) in cerebellar cultures did not express p39.

Analysis of a series of transverse adult cerebellar sections showed that p39-immunoreactive Purkinje cells form an array of parasagittal stripes that encompass both the vermis and hemispheres of most lobules (Fig. 4). The pattern of Purkinje cell p39 immunoreactivity was symmetrical about the midline and reproducible among individual mice ($n=6$). In the anterior lobe vermis, immunoreactive Purkinje cells form narrow stripes separated by broad bands of unstained cells (Fig. 4A). Moving caudally through the vermis (to lobule VI), these stripes disappeared as most of the Purkinje cells became immunoreactive (Fig. 4B–D). In the posterior lobe, a pattern of parasagittal stripes reemerged, with broad p39-immunoreactive stripes and narrower unreactive bands (Fig. 4 D–F). In lobule X, the unreactive stripes again disappeared to leave a uniform array of p39-immunoreactive Purkinje cells (Fig. 4D–F; these antero-posterior changes in p39 immunoreactivity can be further observed in Fig. 7, right panel). In the hemispheres, a p39 immunostaining pattern similar to that in the vermis was observed with alternating stripes of p39-immunoreactive cells in crus I (Fig. 4A, B) and crus II (Fig. 4D, E), and uniformly p39-positive Purkinje cells in the paraflocculi and flocculi (Fig. 4A–C).

Since p39 is closely related to p35 and both are known activators of Cdk5, we compared the cerebellar distribution of these three proteins in the adult mouse (Fig. 5). Cdk5, p39, and p35 were all immunolocalized in Purkinje cells: strongly for Cdk5 and p39, and weakly for p35. Consistent with this, Western blot analysis also showed that p39 is the more abundant Cdk5 activator in the adult cerebellum (Fig. 5A, inset). As described above, p39 expression was restricted to a subset of Purkinje cells forming a parasagittal band pattern (Fig. 5D, G). In contrast, Cdk5 (Fig. 5E, H) and p35 (Fig. 5F, I) were expressed uniformly by Purkinje cells and no parasagittal band pattern of immunostaining was observed. Both p39 and Cdk5 were observed in Purkinje cell somata and dendrites. The punctate immunoreactivity of Cdk5 in the molecular layer represents dense deposits of reaction product in Purkinje cell dendritic profiles, and may include contributions from the neurites of stellate and basket cells (Fig. 5H, K) but p35 staining showed localization only in Purkinje cell somata (Fig. 5I, L).

Expression of p39 in the developing mouse cerebellum

We then sought to investigate the temporal and spatial localization of the p39 protein at different stages of postnatal cerebellar development (Fig. 6). Immunostaining for p39 was first apparent in Purkinje cells from P10, particularly in the posterior lobe vermis. By P15, p39 was observed extensively with stronger immunoreactivity in Purkinje cells. Immunoreactivity of p39 appeared as arrays of parasagittal bands extending through most lobules in both the vermis and hemispheres. This pattern is similar to that observed in the adult cerebellum. On the other hand, granule-cell p39 immunoreactivity developed gradually and uniformly, along with the maturation of the granular layer (data not shown).

Cerebellar expression of p39 corresponds to that of zebrin II

As p39 stripes appear to be similar to that of zebrin II, a side-by-side analysis of p39 and zebrin II staining was performed in serial pairs of transverse sections of the cerebellum. The cerebellar vermis immunolocalization pattern of p39 (Fig. 7, right panel) has a close resemblance to that of zebrin II (Fig. 7, left panel). In the anterior zone, both p39 and zebrin II were observed in Purkinje cells that form narrow parasagittal stripes. In the rostral part of lobule VI, along the posterior face of the primary fissure, the pattern of zebrin II expression changed such that the narrow immunoreactive stripes were replaced by broad bands of zebrin II/aldolase C immunoreactive Purkinje cells (Fig. 7G) that eventually fuse to form a continuous sheet. The same was seen with p39 (Fig. 7H). Alternating stripes of zebrin II/aldolase C and p39 immunoreactivity reappeared in lobule VII (Fig. 7I and J, respectively). In lobule VIII, wide bands of zebrin II and p39-immunoreactive Purkinje cells were clearly separated by non-immunoreactive bands of cells, and in lobule IX, the zebrin II and p39 bands both widened until most Purkinje cells in the nodular zone were immunoreactive for both proteins. Analysis for zebrin II and p39 (Fig. 7Q and R, respectively) immunoreactivity in the hemispheres likewise revealed a similar correspondence between the patterns of expression of both proteins. Double-immunofluorescence staining for zebrin II and p39 supports this interpretation. For example, as shown in Fig. 8, the P4+ band of lobule VIII (Fig. 8A–C) and the P1+/P1-/P2+ region of lobule IX (Fig. 8D–F) showed complete coincidence of zebrin II and p39 immunoreactivity. The possibility of cross-reactivity between p39 and the zebrin II antibody and zebrin II with the p39 antibody was ruled out by Western blot analyses of purified proteins (data not shown).

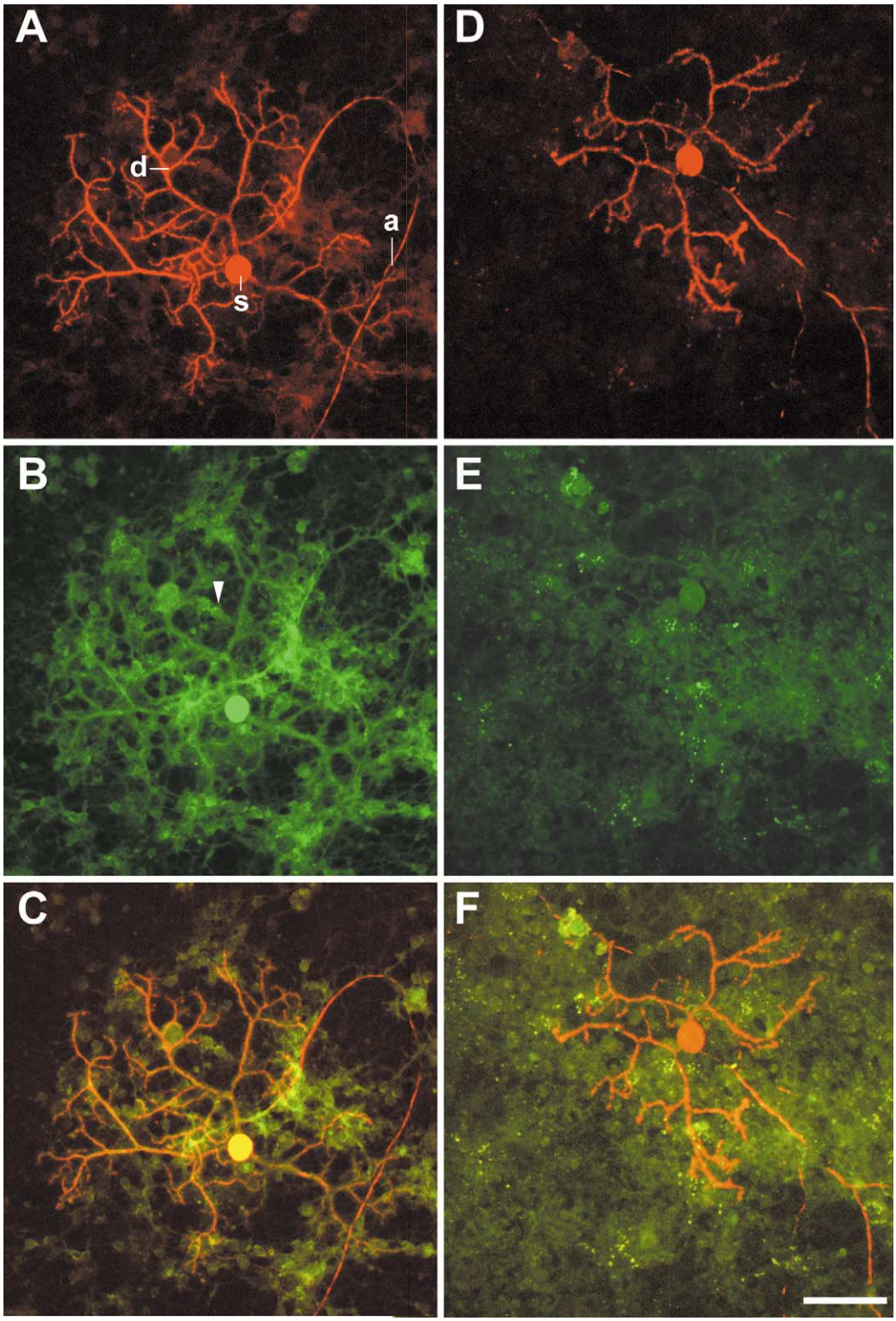

Fig. 3. p39 expression in primary cerebellar culture. After 21 days *in vitro*, dissociated newborn cerebellar cells were double labeled with calbindin (red) and p39 (green). (A and D) The somata (s), dendrites (d) and putative axons (a) of Purkinje cells were immunopositive for calbindin. (B) p39 immunoreactivity was also observed in the Purkinje cell soma and dendrites, and numerous other small neurons resembling granule cells (arrowhead). (C and F) Superimposed images of A and B, and D and E show that p39 is expressed strongly in the Purkinje cell soma and dendrites, and is weak or absent from the axon. The right panel (D–F) shows a Purkinje cell that was not immunopositive for p39 (E and F). Scale bar in F = 50 μm.

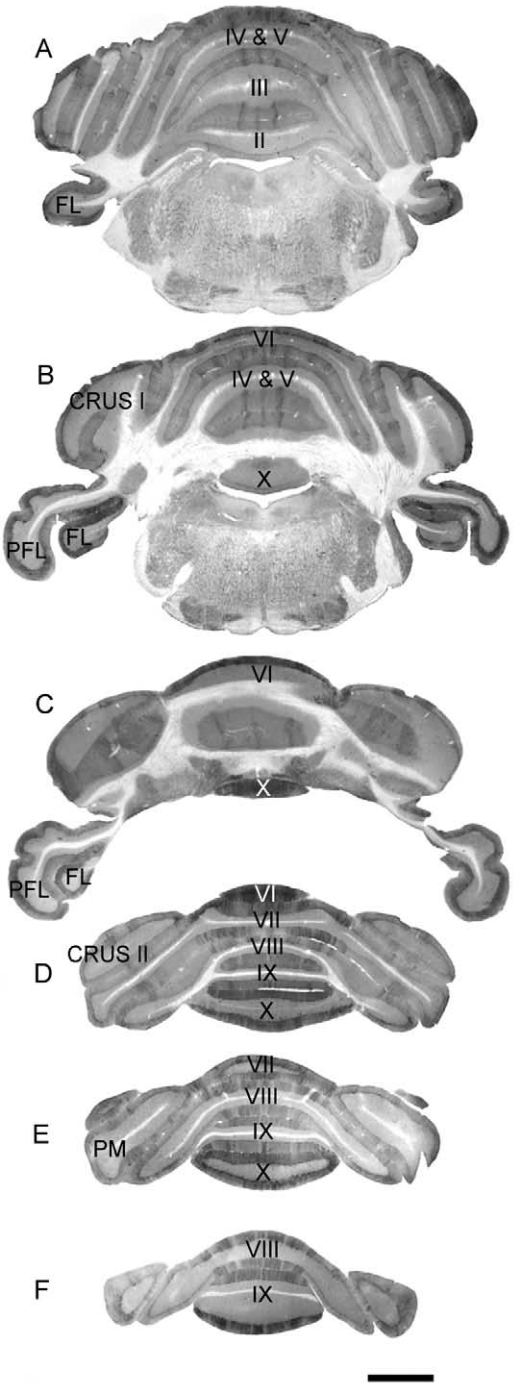

Fig. 4. Distribution of p39-immunoreactive Purkinje cells in the adult mouse cerebellum. A series of transverse sections from the rostral (A) to the caudal (F) cerebellar region was immunostained with p39. Individual lobules in the vermis are indicated in roman numerals. In the hemispheres, Crus I and II, the paramedian lobule (PM), the flocculus (FL) and paraflocculus (PFL) are labeled. Scale bar=1 mm.

Expression of zebrin II in p39−/− mouse cerebellum

Although Cdk5 has been clearly implicated in cerebellar development, and p39 expression is restricted to zones and stripes, the temporal expression profile does not suggest any role for p39 in the development of cerebellar patterning, but rather points to a function in the adult cerebellum. To investigate this more closely, the patterning of the cerebellum in p39−/− mice was analyzed by using anti-zebrin II whole-mount immunocytochemistry. As illustrated in Fig. 8C, cerebellar patterning in p39−/− is normal.

DISCUSSION

The existence of two Cdk5 activators, p35 and its isoform, p39, suggests that each subserves specific cellular functions of Cdk5. This assumption is supported by the mRNA localization during brain development, which revealed distinct temporal and spatial distributions of p35 and p39. A detailed *in situ* hybridization characterization of the temporal and spatial distribution of the p39 transcript during development showed that p39 mRNA is present in neurons from all regions of the developing rat brain (Cai et al., 1997). While p39 mRNA is low in the newborn rat brain, it is heavily expressed in the 3-week-old brain, and in the adult brain, p39 mRNA levels decrease in most neurons, except for the Purkinje cells and granule cells of the cerebellum (Cai et al., 1997). However, in a similar study, p39 mRNA was not detected in granule cells of the adult rat (Zheng et al., 1998) but rather, the transcript was found in Purkinje cells and neurons of the deep cerebellar nuclei (Zheng et al., 1998). The latter study agrees with immunohistochemical studies of p39 in which the protein was also detected in Purkinje cells but not in granule cells of the adult rat brain (Honjyo et al., 1999). However, in the latter study, p39 was also detected in astrocytes and oligodendrocytes.

In the present study, we developed a p39-specific antibody and used it to determine the specific localization of p39 in the developing and adult mouse cerebellum. Our data are consistent with reports on the presence of p39 transcript (Cai et al., 1997; Zheng et al., 1998) and protein (Honjyo et al., 1999) in cerebellar Purkinje cells, granule cells and neurons of the cerebellar nuclei. However, in contrast to some previous findings (Honjyo et al., 1999), no p39 staining was observed in glial cells. Discrepancies among observations may be attributed to the use of different antibodies (e.g. using C-terminal versus N-terminal peptides of p39 for antibody generation).

Unlike its isoform, p35, which is constantly expressed in the neuronal cell body throughout brain development (Tomizawa et al., 1996; Cai et al., 1997; Matsushita et al., 1996), we found that p39 is highly expressed in Purkinje cell somata and dendritic arbors, and rarely and weakly expressed in Purkinje cell axons in the adult mouse cerebellum. Immunolocalization of p39 in Purkinje cell somata and dendrites coincides with that of Cdk5, suggesting that p39 may act as the Cdk5 activator in the mature cerebellum. The absence of p39 immunoreactivity in axons could possibly be due to anterograde transport to the axon terminals perhaps to regulate dynamic remodeling of the Purkinje cell corticonuclear projection. Such a role would

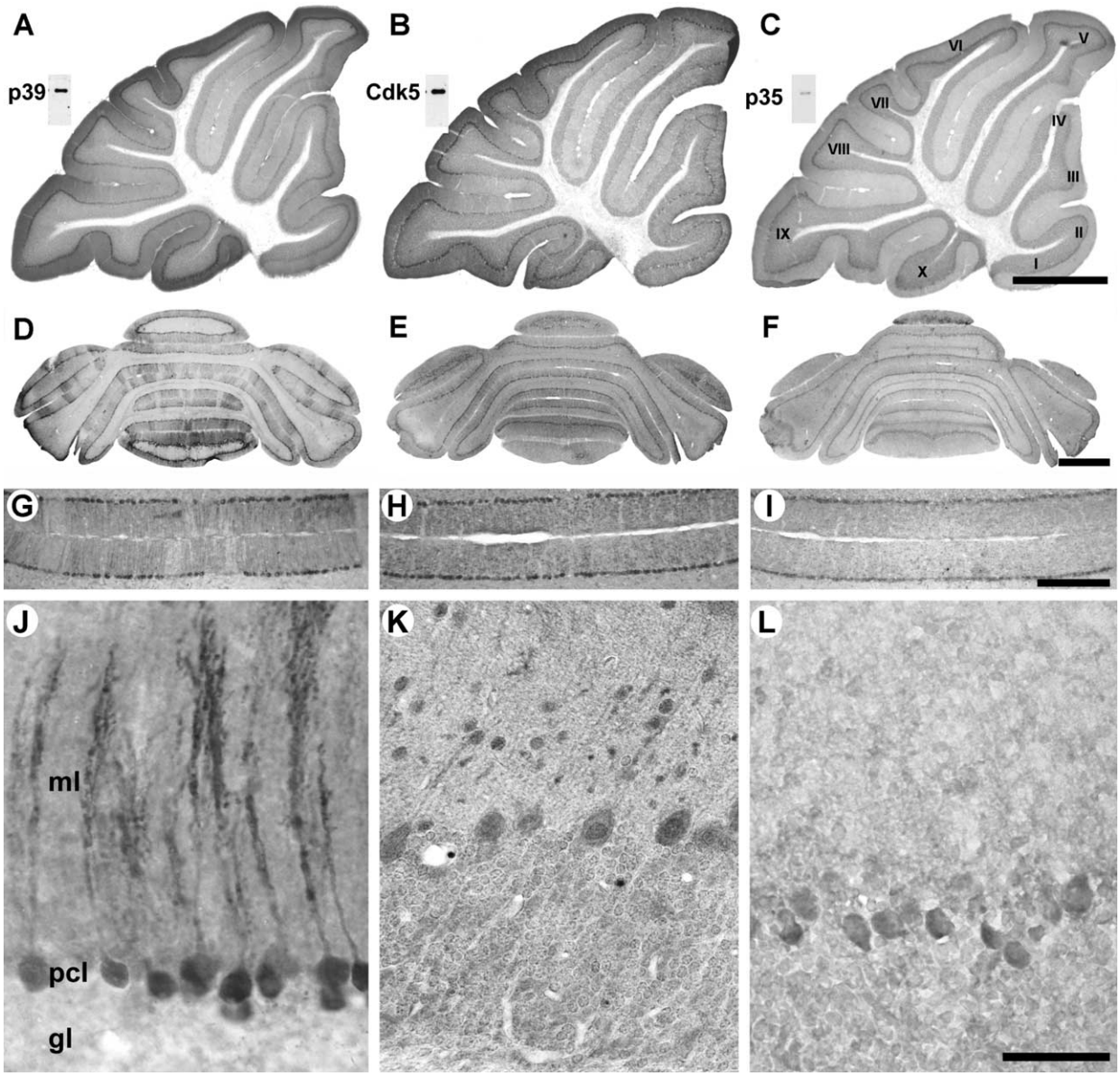

Fig. 5. Comparison of the cerebellar distribution of p39, Cdk5, and p35. Serial sagittal (A–C) and transverse (D–L) cerebellar sections were immunostained for p39 (A, D, G, J), Cdk5 (B, E, H, K), and p35 (C, F, I, L). A higher resolution of lobule IX immunoreactivity to p39 (G, J), Cdk5 (H, K), and p35 (I, L) is also presented. Individual lobules are designated by roman numerals in C. Scale bar for: A–F=1 mm, G–I=500 μm, J–L=50 μm. Adult mouse cerebellar extracts (50 μg) were also subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot analysis using a p39 (A, inset), Cdk5 (B, inset), or p35 (C, inset) specific antibody. The blots were developed under the same conditions.

be consistent with the observation that a fraction of neuronal p39 is associated with synaptic junctions (Humbert et al., 2000), and the suggestion that Cdk5 may coordinate general axon patterning events (Connell-Crowley et al., 2000). However, the lack of significant phenotypic alterations in p39−/− mice (Ko et al., 2001) suggests that p35 may compensate for p39 activity in the cerebellum. Indeed, Cdk5−/− mice exhibit severe brain defects and perinatal mortality (Ohshima et al., 1996), but p35−/− mice display less brain defects and survive for about 6 weeks, and p35 and p39 double-knockout mice exhibit a phenotype similar to that of Cdk5−/− mice (Ko et al., 2001), suggesting that p35 and p39 compensate each other to activate Cdk5. Nonetheless, it appears that in the normal adult cerebellum, p39 is primarily responsible for Cdk5 activity.

In this study, we further found that expression of the p39 protein is confined to a subset of cerebellar Purkinje cells. Cells that are immunopositive for p39 form a symmetrical, reproducible array of parasagittal bands in both the vermis and the hemispheres. As p39 shows an almost complete colocalization with zebrin II/aldolase C, its distribution differs from other compartmentation antigens in the cerebellum such as L7/pcp-2-*lacZ* (Ozol et al., 1999) or p-path (Leclerc et al., 1992). Nonetheless, it now seems that p39 may also serve as an intrinsic marker to further

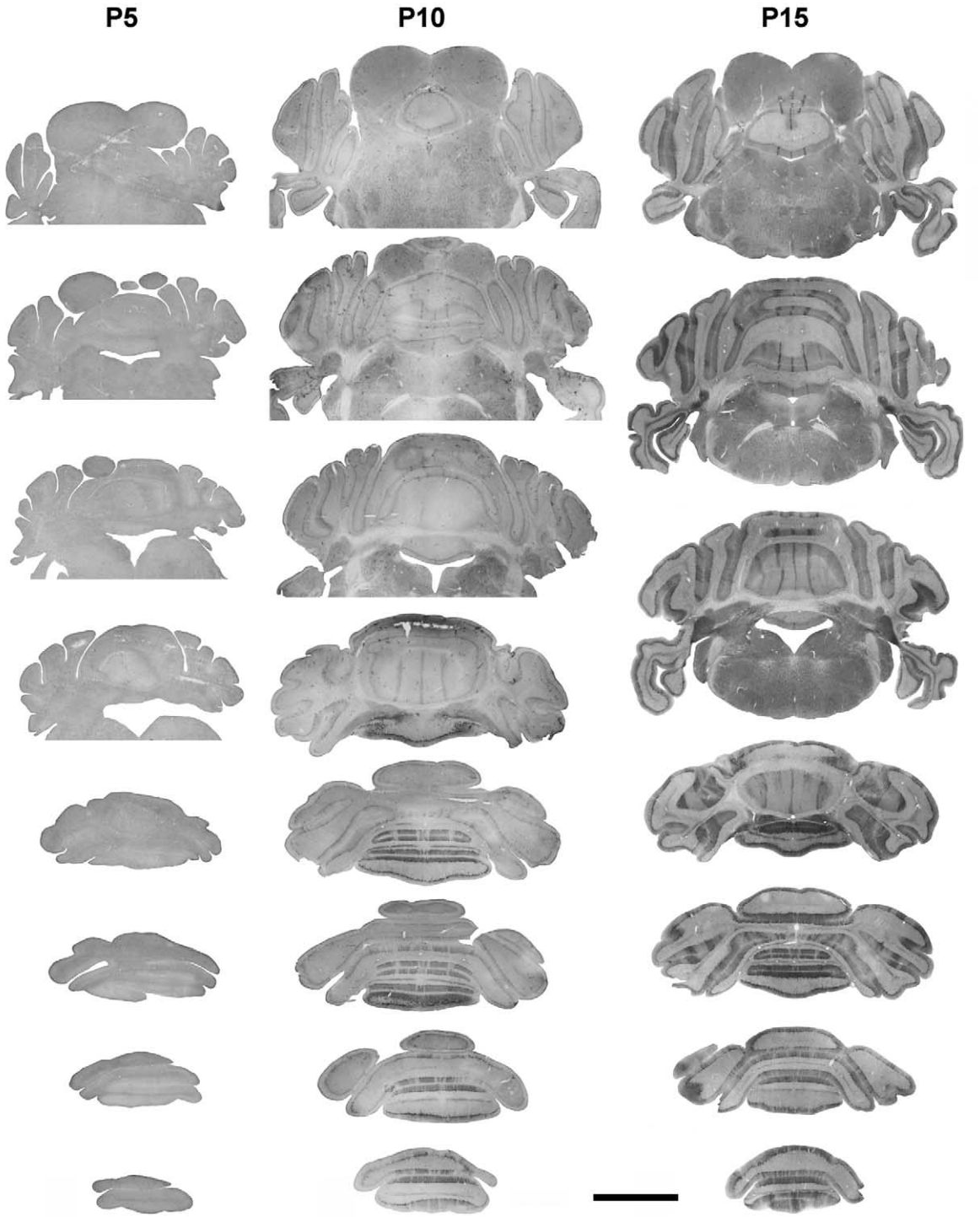

Fig. 6. Expression of p39 in the developing postnatal mouse cerebellum. A series of rostral (top) to caudal (bottom) transverse cerebellar sections from postnatal day (P) 5, P10, and P15 mice were immunostained for p39. Scale bar=2 mm.

analyze the structural, molecular and functional aspects of the cerebellum. Our finding that zebrin II patterning is normal in p39−/− mice indicates that p39 does not have a role in the development of cerebellar compartmentation. Rather, it seems likely that p39 plays a role in normal cerebellar function. Zones and stripes of Purkinje cells are tightly correlated with the terminal fields of the cerebellar afferents—both climbing fibers (Gravel et al., 1987) and mossy fibers (Gravel and Hawkes, 1990; Sotelo and Wassef, 1991; Ji and Hawkes, 1994; Akintunde and Eisenman, 1994)—as mapped by using anterograde tracing. Similarly, afferent terminal fields as reflected by tactile recep-

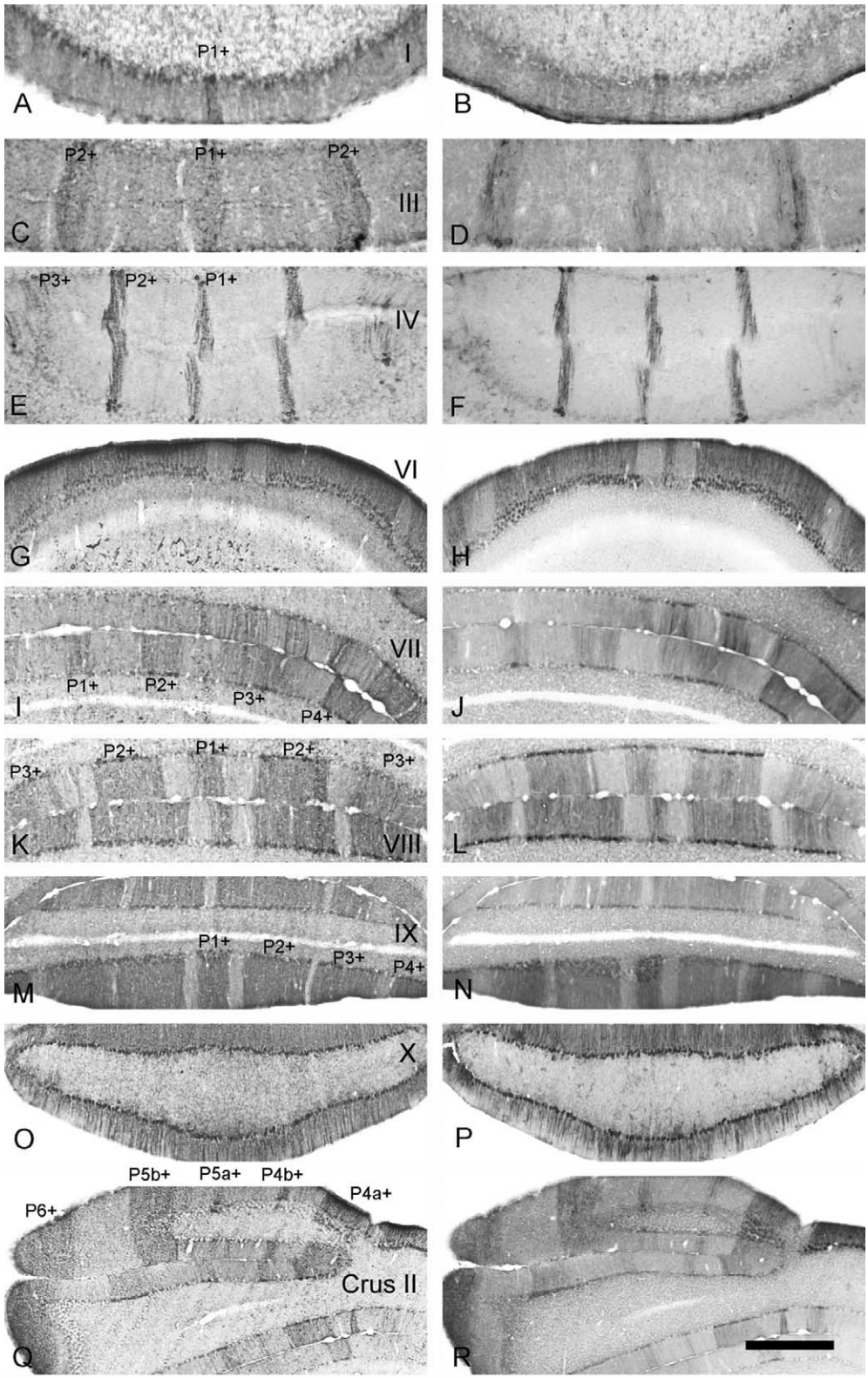

Fig. 7. Comparison of the expression pattern of p39 and zebrin II in the adult mouse cerebellum. Serial pairs of transverse sections of the cerebellum were immunostained for either zebrin II (left panel) or p39 (right panel). Individual lobules are labeled in roman numerals. Individual zebrin II-immunoreactive bands of Purkinje cells are designated P1+ to P6+ according to Eisenman and Hawkes (1993). The pairs of panels are arranged from anterior to posterior: lobules I–V of the anterior zone (A–F), the anterior portion of the central zone (G, H), the posterior zone (I–N), the nodular zone (O, P), Crus II of the hemisphere (Q, R). Scale bar=500 μm.

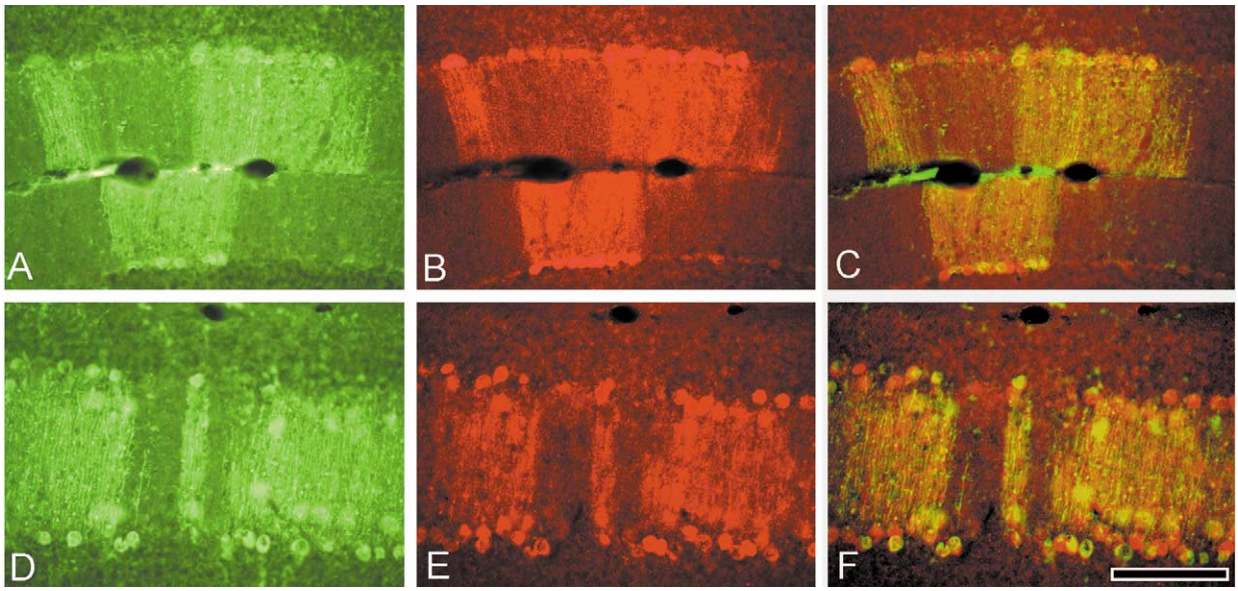

Fig. 8. Colocalization of zebrin II and p39 by immunofluroscence staining. Transverse cerebellar sections were double stained for zebrin II (green) and p39 (red). A–C represents zebrin II P4+ in lobule VIII; D–F represents P1+/P1−/P2+ in lobule IX. C and F represent superimposed images of the preceding two panels. Scale bar=100 μm.

tive field maps also bear a constant, if complex relationship to the Purkinje cell topography (Chockkan and Hawkes, 1994; Chen et al., 1996; Hallem et al., 1999). The anatomy of the cerebellar cortical efferent projections, the axons of the Purkinje cells terminating in the cerebellar and vestibular nuclei, is less well understood, but at least a coarse topographical mapping is clearly present (e.g. fastigial). Thus, the expression domains revealed by p39 immunocytochemistry correlate with the functional compartmentation of the cerebellar cortex. Indeed, it is conceivable that p39 interacts with zebrin II/aldolase C to regulate functional compartmentation in the cerebellum, possibly fine-tuning the glycolytic activity between stripes to match the patterns of sensory or motor activity.

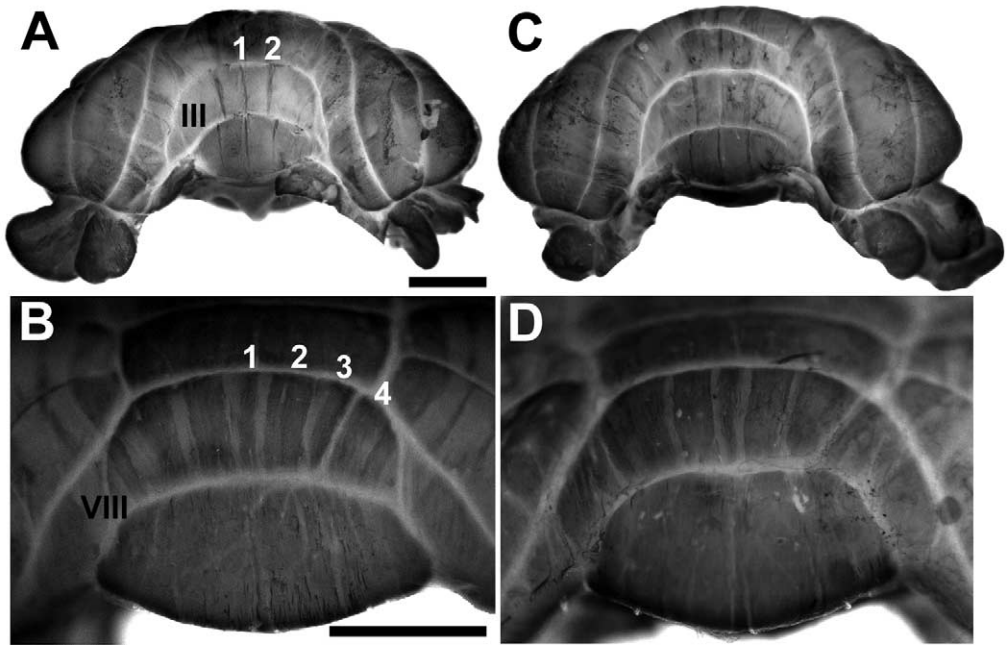

Fig. 9. Whole-mount zebrin II expression in the cerebellum of p39−/− mice. Anterior view of the AZ in a wild-type (A) and p39−/− (C) mouse. Posterior view of the PZ in a wild type (B) and p39−/− (D) mouse. The P1+ to P4+ stripes (designated in Arabic numerals), and lobules III and VIII are labeled. Scale bars=1 mm. Scale bar in A applies to C, and the scale bar in B applies to D.

Acknowledgements—We thank Estrella Gonzales for her technical assistance. This study was supported in part by operating grants from the KOSEF (Y.G.J.) and the Canadian Institutes of Health Research (R.H., K.-Y.L.). Ki-Young Lee is an Alberta Heritage Foundation for Medical Research (AHFMR) Scholar.

REFERENCES

Ahn AH, Dziennis S, Hawkes R, Herrup K (1994) The cloning of zebrin II reveals its identity with aldolase C. Development 120: 2081–2090.

Akintunde A, Eisenman LM (1994) External cuneocerebellar projections and Purkinje cell zebrin II bands: a direct comparison of parasagittal banding in the mouse cerebellum. J Chem Neuroanat 7:75–86.

Armstrong CL, Hawkes R (2000) Pattern formation in the cerebellar cortex. Biochem Cell Biol 78:551–562.

Brochu G, Maler L, Hawkes R (1990) Zebrin II: a polypeptide antigen expressed selectively by Purkinje cells reveals compartments in rat and fish cerebellum. J Comp Neurol 291:538–552.

Cai XH, Tomizawa K, Tang D, Lu YF, Moriwaki A, Tokuda M, Nagahata S, Hatase O, Matsui H (1997) Changes in the expression of novel Cdk5 activator messenger RNA (p39 mRNA) during rat brain development. Neurosci Res 28:355–360.

Celio MR (1990) Calbindin D-28k and parvalbumin in the rat nervous system. Neuroscience 35:375–475.

Chen G, Hanson CL, Ebner TJ (1996) Functional parasagittal compartments in the rat cerebellar cortex: an in vivo optical imaging study using neutral red. J Neurophysiol 76:4169–4174.

Chockkan V, Hawkes R (1994) Functional and antigenic maps in the rat cerebellum: zebrin compartmentation and vibrissal receptive fields in lobule IXa. J Comp Neurol 345:33–45.

Connell-Crowley L, Le Gall M, Vo DJ, Giniger E. The cyclin-dependent kinase Cdk5 controls multiple aspects of axon patterning *in vivo*. Curr Biol 10:599–602.

Davis CA (1993) Whole-mount immunohistochemistry. Methods Enzymol 225:502–516.

Dent JA, Paulson AG, Klymkowsky MW (1989) Whole-mount immunocytochemical analysis of the expression of the intermediate filament protein vimentin in Xenopus. Development 105:61–74.

Eisenman LM, Hawkes R (1993) Antigenic compartmentation in the mouse cerebellar cortex: zebrin and HNK-1 reveal a complex, overlapping molecular topography. J Comp Neurol 335:586–605.

Fletcher AI, Shuang R, Giovannucci DR, Zhang L, Bittner MA, Stuenkel EL. Regulation of exocytosis by cyclin-dependent kinase 5 via phosphorylation of Munc18. J Biol Chem 274:4027–4035.

Gravel C, Eisenman LE, Sasseville R, Hawkes R (1987) Parasagittal organization of the rat cerebellar cortex: a direct correlation between antigenic Purkinje cell bands revealed by mabQ113 and the organization of the olivocerebellar projection. J Comp Neurol 263: 294–310.

Gravel C, Hawkes R (1990) Parasagittal organization of the rat cerebellar cortex: direct comparison of Purkinje cell compartments and the organization of the spinocerebellar projection. J Comp Neurol 291:79–102.

Hallem JS, Thompson J, Gundappa-sulur S, Hawkes R, Bjaallie JG, Bower JM (1999) Spatial correspondence between tactile projection patterns and the distribution of the antigenic Purkinje cell markers anti-zebrin I and anti-zebrin II in the cerebellar folium crus IIa of the rat. Neuroscience 93:1083–1094.

Hawkes R, Eisenman LM (1997) Stripes and zones: the origins of regionalization of the adult cerebellum. Perspect Dev Neurobiol 5:95–104.

Herrup K, Kuemerle B (1997) The compartmentalization of the cerebellum. Annu Rev Neurosci 20:61–90.

Homayouni R, Curran T (2000) Cortical development: Cdk5 gets into sticky situations. Curr Biol 10:R331–R334.

Honjyo Y, Kawamoto Y, Nakamura S, Nakano S, Akiguchi I (1999) Immunohistochemical localization of Cdk5 activator p39 in the rat brain. Neuroreport 10:3375–3379.

Humbert S, Lanier LM, Tsai LH (2000) Synaptic localization of p39, a neuronal activator of Cdk5. Neuroreport 11:2213–2216.

Ji Z, Hawkes R (1994) Topography of Purkinje cell compartments and mossy fiber terminal fields in lobules II and III of the rat cerebellar cortex: spinocerebellar and cuneocerebellar projections. Neuroscience 61:935–954.

Ko J, Humbert S, Bronson RT, Takahashi S, Kulkarni AB, Li E, Tsai LH (2001) p35 and p39 are essential for cyclin-dependent kinase 5 function during neurodevelopment. J Neurosci 21:6758–6771.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Leclerc N, Schwarting G, Herrup K, Hawkes R, Yamamoto M (1992) Compartmentation in mammalian cerebellum: zebrin II and P-Path antibodies define three classes of sagittally organized bands of Purkinje cells. Proc Natl Acad Sci USA 89:5006–5010.

Lee KY, Clark AW, Rosales JL, Chapman K, Fung T, Johnston RN (1999) Elevated neuronal Cdc2-like kinase activity in the Alzheimer disease brain. Neurosci Res 34:21–29.

Nilden F, Backstrom A, Bark C (1998) Molecular cloning and characterization of a mouse gene encoding an isoform of the neuronal cyclin-dependent kinase 5 (CDK5) activator. Biochem Biophys Acta 1398:371–376.

Lew J, Huang QQ, Qi Z, Winkfein RJ, Aebersold R, Hunt T, Wang JH (1994) A brain-specific activator of cyclin-dependent kinase 5. Nature 371:423–426.

Matsushita M, Tomizawa K, Lu YF, Moriwaki A, Tokuda M, Itano T, Wang JH, Hatase O, Matsui H (1996) Distinct cellular compartment of cyclin-dependent kinase 5 (Cdk5) and neuron-specific Cdk5 activator protein (p35) in the developing rat cerebellum. Brain Res 734:319–322.

Morgan DO (1997) Cyclin-dependent kinases: engines, clocks, and microprocessors. Annu Rev Cell Dev Biol 13:261–291.

Nilden F, Backstrom A, Bark C (1998) Molecular cloning and characterization of a mouse gene encoding an isoform of the neuronal cyclin-dependent kinase 5 (CDK5) activator. Biochem Biophys Acta 1398:371–376.

Oberdick J, Baader SL, Schilling K (1998) From zebra stripes to postal zones: deciphering patterns of gene expression in the cerebellum. Trends Neurosci 21:383–390.

Ohshima T, Ogawa M, Veeranna, Hirasawa M, Longenecker G, Ishiguro K, Pant HC, Brady RO, Kulkarni AB, Mikoshiba K (2001) Synergistic contributions of cyclin-dependant kinase 5/p35 and Reelin/Dab1 to the positioning of cortical neurons in the developing mouse brain. Proc Natl Acad Sci USA 98:2764–2769.

Ohshima T, Ward JM, Huh CG, Longenecker G, Veeranna, Pant HC, Brady RO, Martin LJ, Kulkarni AB (1996) Targeted disruption of the cyclin-dependent kinase 5 gene results in abnormal corticogenesis, neuronal pathology and perinatal death. Proc Natl Acad Sci USA 93:11173–11178.

Ozol K, Hayden JM, Oberdick J, Hawkes R (1999) Transverse zones in the vermis of the mouse cerebellum. J Comp Neurol 412:95–111.

Patrick GN, Zukerberg L, Nikolic M, de la Monte S, Dikkes P, Tsai LH (1999) Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration. Nature 402:615–622.

Paudel HK (1997) Phosphorylation by neuronal cdc2-like protein kinase promotes dimerization of Tau protein in vitro. J Biol Chem 272:28328–28334.

Paudel HK, Lew J, Ali Z, Wang JH (1993) Brain proline-directed proteinkinase phosphorylates tau on sites that are abnormally phosphorylated in tau associated with Alzheimer's paired helical filaments. J Biol Chem 268:23512–23518.

Sillitoe RV, Hawkes R (2002) Whole-mount immunohistochemistry: a high-throughput screen for patterning defects in the mouse cerebellum. J Histochem Cytochem 50:235–244.

Sotelo C, Wassef M (1991) Cerebellar development: afferent organization and Purkinje cell heterogeneity. Philos Trans R Soc Lond B Biol Sci 331:307–313.

Tang D, Yeung J, Lee KY, Matsushita M, Matsui H, Tomizawa K, Hatase O, Wang JH (1995) An isoform of the neuronal cyclin-dependent kinase 5 (Cdk5) activator. J Biol Chem 270:26897–26903.

Tomizawa K, Matsui H, Matsushita M, Lew J, Tokuda M, Itano T, Konishi R, Wang JH, Hatase O (1996) Localization and developmental changes in the neuron-specific cyclin-dependent kinase 5 activator (p35) in the rat brain. Neuroscience 74:519–529.

Tsai LH, Delalle I, Caviness VS, Chae T, Harlow E (1994) p35 is a neural-specific regulatory subunit of cyclin-dependent kinase 5. Nature 371:419–423.

Uchida T, Ishiguro K, Ohnuma J, Takamatsu M, Yonekura S, Imahori K (1994) Precursor of Cdk5 activator, the 23 kDa subunit of tau protein kinase II: its sequence and developmental change in brain. FEBS Lett 355:35–40.

Wu DC, Yu YP, Lee NT, Yu AC, Wang JH, Han YF (2000) The expression of Cdk5, p35, p39 and Cdk5 kinase activity in developing, adult, and aged rat brains. Neurochem Res 25:923–929.

Zheng M, Leung CL, Liem RKH (1998) Region-specific expression of cyclin-dependent kinase 5 (Cdk5) and its activators, p35 and p39, in the developing and adult rat central nervous system. J Neurobiol 35:141–159.

(Accepted 3 December 2002)